10x Genomics®

Sample Preparation Demonstrated Protocol

Fresh Frozen Human-Mouse Cell Line Mixtures for Single Cell RNA Sequencing

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Manual Part Number

CG00014 Rev E

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For technical information or advice, please contact our Customer Technical Support Division online at any time.

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Demonstrated Protocol

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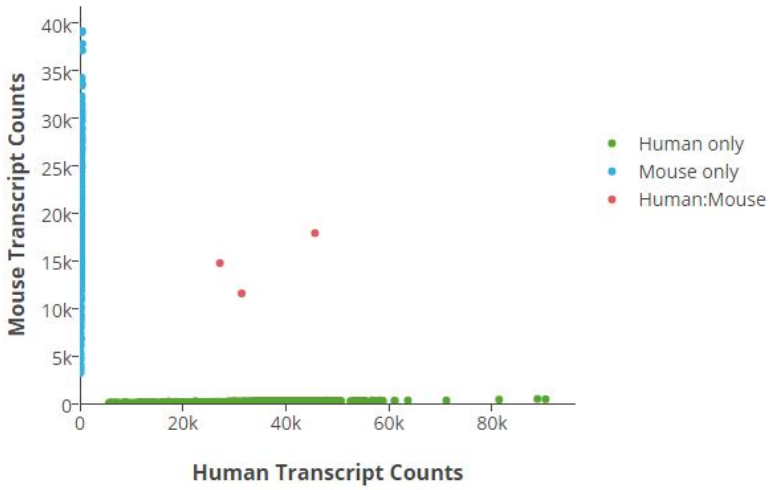
1. Overview

The ability of the 10x Genomics® Single Cell Solutions to partition single cells in a heterogeneous population can be verified by profiling a mixture of cells from two different species. Ideally, all sequence reads from a single Gel Bead-in-EMulsion (GEM) will be unambiguously mapped to the transcriptome of only one of the two species. The fraction of GEMs containing a mixture of human and mouse transcripts is used to infer doublet rates (below, red circles).



Approximately 1000 cells from a 1:1 mixture of human (293T/17) and mouse (NIH/3T3) cells were profiled using the Chromium™ Single Cell 3' Solution (v2). 99.7% of the cell-containing GEMs resulted in sequencing reads mapping to only one species. This implies a total doublet rate (including human:human and mouse:mouse doublets) of 0.6% in this particular experiment.

10x Genomics routinely uses a 1:1 mixture of human and mouse cells to validate the technical performance of the 10x Genomics Single Cell Solutions. For convenience, cell mixtures are cryopreserved in large batches, thawed when needed, and then washed and counted prior to loading into the system without the need for further cell culturing.

This Demonstrated Protocol outlines (i) how to generate cryopreserved 1:1 mixtures of human and mouse cells and (ii) how to thaw, wash, and count cryopreserved 1:1 mixtures of human and mouse cells in preparation for use in 10x Genomics Single Cell Protocols.

2. Getting Started

2.1. Tips & Safety

Best practices for handling any cell line include using sterile technique, nuclease-free reagents and consumables, minimizing pipetting steps, and using wide-bore pipette tips where possible to minimize cell damage.

To determine accurate cell counts, best practices include sampling the cell suspension at least twice and at least two counts on each sample (*i.e.* a minimum of four counts in total, based on two independent draws from the cell suspension).

CRITICAL!

*293T/17 cells contain Adeno and SV-40 viral DNA sequences, which requires classification at **Biosafety Level 2** based on U.S. Public Health Service Guidelines. NIH/3T3 cells are classified at Biosafety Level 1. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage and disposal of biological materials.*

2.2. General Materials

Supplier	Description	Part Number (US)
-	Refrigerated Benchtop Centrifuge for 15 ml and 50 ml tubes	-
	Microcentrifuge for 2 ml LoBind tubes	-
	Heated Water Bath, 2l	-
Rainin	Tips LTS 1ML Filter RT-L1000FLR	17007954
	Tips LTS W-O 1ML Fltr RT-L1000WFLR	17014297
	Pipet-Lite LTS Pipette L-1000XLS+	17014382
Sigma-Aldrich	Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin (alternative to Thermo Fisher product)	SRE0036
Miltenyi	MACS SmartStrainers, 30 µm	130-098-458
Thermo Fisher Sci	UltraPure™ Bovine Serum Albumin (BSA, 50 mg/ml) (alternative to Sigma-Aldrich product)	AM2616
	Trypan Blue Stain (0.4%) for use with the Countess® Automated Cell Counter	T10282
	Countess® II Automated Cell Counter	AMQAX1000
	Countess® II Automated Cell Counting Chamber Slides	C10228
	Gibco 0.25% Trypsin-EDTA (1X), phenol red	25200056
	Nunc™ Biobanking and Cell Culture Cryogenic Tubes, 1.8 ml	368632
	Dimethyl Sulfoxide (DMSO), for molecular biology	AC327182500
Eppendorf	DNA LoBind Tubes, 2.0 ml*	022431048
Integra	PIPETBOY acu 2	155018
VWR	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 50 ml	82018-050
	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 15 ml	21008-103
	10 ml Serological Pipette	89130-898
	Seradigm Premium Grade Fetal Bovine Serum (FBS)	97068-085
	75 cm^2 Cell Culture Flask, Canted Neck	46610-078
ATCC	DMEM	30-2002
Biocision	CoolCell® FTS30 Cell Freezing Container	BSC-170

*No substitutions are allowed. Items have been validated by 10x Genomics® and are required for Single Cell workflow, training and system operations.

2.3. Specific Cell Sourcing & Culturing

a) Obtain vials of cryopreserved human 293T/17 (CRL-11268) and murine NIH/3T3 (CRL-1658) cells from ATCC or a licensed local distributor. Culture the cells as recommended by the distributor.

NOTE

293T/17 cells grow to a higher density than NIH/3T3 cells, and NIH/3T3 cells will often be the limiting reagent in this part of the Protocol. Approximately 5 times more NIH/3T3 culture flasks are required to achieve balanced cell numbers (e.g., 4 confluent T75 flasks of 293T/17s will require ~20 nearly confluent T75 flasks of NIH/3T3 cells).

2.4. Preparation – Buffers & Media

a) Prepare 1 ml chilled (4°C) cryopreservation medium (DMEM + 20% FBS + 10% DMSO) for every 10^6 cells to be cryopreserved.

b) Chill a CoolCell® FTS30 or equivalent cell freezing container in a 4°C refrigerator.

3. Cryopreservation of Human-Mouse Cell Mixtures

3.1. Cryopreservation Protocol

a) Remove medium from cultured 293T/17 and NIH/3T3 cells, rinse culture vessels, detach cells using trypsin, spin down cells, resuspend cell pellets in growth medium by pipetting up and down 10 times, and strain cells to remove clumps and ensure single cell suspensions. (Consult Sample Preparation Demonstrated Protocol *Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing* (CG00054) for more details on harvesting adherent cells).

b) Using a Countess® II Automated Cell Counter, count the 293T/17 and NIH/3T3 single cell suspensions after cell straining and measure the total volume of each cell culture. If the concentration is too high to achieve an accurate cell count, further dilute the cells with growth medium, thoroughly mix the resulting cell suspension, and recount. An accurate count for each cell type is important.

c) Based on total volumes (V) and concentrations (C), calculate total cell number (N = C x V) for each cell type to determine which cell type is limiting.

Example:

$V_{293T/17}$ = 10 ml $C_{293T/17}$ = 3 x 10^6 cells/ml $N_{293T/17}$ = 3 x 10^7 cells

$V_{NIH/3T3}$ = 6 ml $C_{NIH/3T3}$ = 2 x 10^6 cells/ml $N_{NIH/3T3}$ = 1.2 x 10^7 cells

NIH/3T3 cells are limiting

d) Determine how much of the non-limiting cell type should be added to the limiting cell type to achieve a 1:1 ratio, then prepare the 1:1 Human-Mouse mixture. Calculate the total cell number (293T/17 + NIH/3T3 cells).

Example continued:

Since NIH/3T3 cells are limiting

$$V_{293T/17} = \frac{N_{NIH/3T3}}{C_{293T/17}} = \frac{1.2 \text{ x } 10^7 \text{cells}}{3 \text{ x } 10^6 \text{ cells/ml}} = 4 \text{ ml}$$

4 ml 293T/17 cells is added to 6 ml of NIH/3T3 cells to generate 10 ml of a 1:1 mixture

The $N_{293T/17+NIH/3T3}$ in this mixture is 2.4 x 10^7 cells

e) Centrifuge the 1:1 mixture at **300 rcf** for **5 min.**

f) Resuspend the pellet in an appropriate volume of cryopreservation medium to achieve an overall cell concentration of 1 x 10^6 cells/ml in the cryovials.

Example continued:

After centrifugation, cells resuspended in 24 ml chilled cryopreservation medium

g) Dispense **1 ml** aliquots of the 1:1 mixture into cryovials.

h) Remove the chilled CoolCell® FTS30 from the 4°C refrigerator and place the cryovials inside.

i) Place the chilled CoolCell® FTS30 in the **–80°C** freezer for at least **4 h**, ensure that the bottom and top vents are not obstructed to allow for adequate air flow.

j) After **4 h**, transfer the cryovials to either liquid nitrogen or vapor-phase nitrogen for long-term storage.

4. Thawing & Washing Human-Mouse Cell Mixtures

4.1. Preparation

a) Warm a water bath to 37°C prior to commencing the thawing protocol.

b) Prepare 1X PBS with 0.04% BSA (400 µg/ml) solution for the final resuspension.

4.2. Thawing, Washing & Counting Cells

a) Remove cryovial(s) from liquid or vapor-phase nitrogen storage and rapidly thaw in the water bath at **37°C** for **2 – 3 min**. Remove from the water bath when a tiny ice crystal remains.

b) Pipette mix the cells and transfer the entire volume to a 2 ml tube.

c) Centrifuge cells at **150 rcf** for **3 min**.

CRITICAL!

Depending on the rotor type, the cell pellet forms on the side or on the bottom of the conical vial. Know the expected position of the pellet, especially when working with small or limited cells, as the pellet can be difficult to see.

d) Using a disposable transfer pipette with fine tip, remove supernatant without disrupting cell pellet.

e) Using a **wide-bore** pipette tip, add **1 ml** 1X PBS with 0.04% BSA to each tube and gently pipette mix 5 times and invert tubes to resuspend cell pellet. Pool the tubes if necessary.

f) Centrifuge cells at **150 rcf** for **3 min**.

g) Remove supernatant without disrupting cell pellet.

Repeat

h) Repeat steps e – g for a total of 2 washes.

i) Using a **regular-bore** pipette tip, add **1 ml** 1X PBS with 0.04% BSA. Gently pipette mix 10 – 15 times or until cells are completely suspended.

NOTE

Do not invert the tube in this step, as cells can stick to the sides of the tube, thereby changing the cell concentration.

j) Determine the cell concentration using a Countess® II Automated Cell Counter. The target cell concentration is ~7 x 10^5 cells/ml (700 cells/µl).

k) Proceed with the 10x Genomics® Single Cell Protocol.